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Sodium carbonate and bicarbonate treatments induce resistance to postharvest green mould on citrus fruit

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ABSTRACT

The aim of this study was to investigate the ability of two salts, sodium carbonate and bicarbonate, to activate defence mechanisms in citrus fruit against postharvest green mould caused by *Penicillium digitatum*. In particular, once there was confirmed salt antifungal activity in the absence of direct contact with the pathogen, changes in enzyme activity and expression levels of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase (PAL), and phytoalexin (scoparone, scopoletin, umbelliferone) and sugar (glucose, fructose, sucrose) contents in treated oranges were analyzed. Overall, sodium carbonate and bicarbonate increases the activity of β-1,3-glucanase, peroxidase, and PAL enzymes in orange tissues. Gene expression analyses confirmed PAL up-regulation particularly 12 h after treatment application. HPLC analyses of peel extracts showed increased amounts of the sugars and phytoalexins, compared to control tissues, with sucrose and scoparone being the most represented. The results suggest that, although salts exert a direct antifungal effect on *P. digitatum*, they are also able to induce citrus fruit defence mechanisms to postharvest decay. The defence response seems correlated with the up-regulation of the phenylpropanoid pathway, which has a role in the adaptation to various stresses. This response could result in natural reaction to wounding and pathogen attack in citrus, enhancing its protective effect. As a consequence, the fruit might have a better chance of successful defence against the decay.

1. Introduction

The most severe postharvest fungal diseases of citrus fruit are green and blue moulds caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer, respectively. Blue mould is important on fruit kept under cold storage, whereas green mould may cause 60–80% of decay under ambient conditions (Moscoso-Ramírez et al., 2013), although numerous factors related to the fruit itself, the pathogen, and the postharvest environment can influence incidence and severity (Youssef et al., 2011). Traditionally, control is performed with synthetic fungicides. However, issues associated with their use, such as risks for human and environmental health, pathogen resistance, costs of registration and re-registration of active ingredients, *etc.*, have motivated the search for new and safer alternatives (Sanzani et al., 2012). In this context, the activity of several organic and inorganic salts has been comprehensively tested at concentrations of 2–6%, on a wide range of commodities including citrus (Smilanick et al., 1999; Palou et al., 2008; Romanazzi et al., 2012). In a previous study, several salts included in the Generally Regarded as Safe (GRAS) category were tested *in vitro* and *in vivo* against Penicillium rots of citrus fruit (Youssef et al., 2012b). Among them, sodium carbonate (SC) and sodium bicarbonate (SB) at 3% (w/v) proved to effectively reduce up to 100% disease incidence on clementines and oranges. However, little is known about their mode of action.

Much of the previous research indicated that one of the main elements underlying the efficacy of SB is the direct activity against the fungus due to its high pH (Smilanick et al., 2005; Venditti et al., 2005; Nigro et al., 2006). However, great differences in control efficacy of *P. digitatum* infections on lemons and oranges were observed among salt solutions with the same pH (Smilanick et al., 1999; Palou et al., 2001). Therefore, other possible mechanisms, such as the induction of host defence responses, might be involved.

There is a considerable interest in exploring the activation of plant defence mechanisms as alternative to traditional control methods (Sharma et al., 2002). In citrus, it has been reported that

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increased resistance against *P. digitatum* infections can be achieved by application of physical means (Kim et al., 1991; Rodov et al., 1992; Droby et al., 1993), safe chemical compounds (Venditti et al., 2005; Ippolito and Sanzani, 2011; Fallanaj et al., 2013), or antagonistic microorganisms (Fajardo et al., 1998; Droby et al., 2002). Resistance inducers are compounds that have a composition based on pathogen or plant constituents, or their analogs, so that they can react with plant receptors and activate plant defences, thus preventing pathogen infections (Terry and Joyce, 2004).

In several plant–pathogen interactions, resistance to infection is correlated with *de novo* synthesis of phytoalexins and pathogenesis-related (PR) proteins (Van Loon et al., 2006). Non-specific stress conditions, both biotic and abiotic, might activate the synthesis of these compounds, normally absent in healthy or unstressed tissues, acting on pathogens mainly by disruption of cell membranes (Smith, 1996).

Some PR proteins, such as β-1,3-glucanases (E.C. 3.2.1.39) belonging to the PR-2 family, and chitinases (E.C. 3.2.1.14) belonging to PR-3, -4, -8 and -11 families (Van Loon et al., 2006), are able to degrade fungal cell wall constituents, namely β-1,3-glucan and chitin, and thus exhibit antifungal properties. Their biosynthesis and accumulation is considered a major defence mechanism (Odjakova and Hadjiivanova, 2001). Within the plant defence strategy, also peroxidases (E.C. 1.11.1.7) play a key role, during the synthesis of lignin, which acts as a cell wall reinforcement enhancing resistance against multiple pathogens, and altering the ability of citrus fruit to cope with *Penicillium* infection through antioxidant activity (Ballester et al., 2006). Finally, phenylalanine ammonia lyase (E.C. 4.3.1.5; PAL) activity, which is the first enzyme in the phenylpropanoid pathway, increases in response to several kinds of stress including wounding (Ke and Saltveit, 1989). In the phenylpropanoid pathway, important compounds such as carbohydrates, either in a free state or as derivatives, are also involved. Apart from playing an important role in fruit quality properties (colour, texture, and flavour), carbohydrates are involved in citrus fruit physiology, for instance providing the substrate for the synthesis of various secondary metabolites including phenylpropanoids (Jackson, 2008).

In the present study, chemical, biochemical, and molecular analyses were carried out in order to evaluate changes in enzyme activity, gene expression levels and phytoalexin and carbohydrate contents in citrus fruit following SC and SB treatments. These results would support their putative role as resistance inducers against *Penicillium* decay of citrus fruit.

2. Materials and methods

2.1. Plant material

Oranges [*Citrus sinensis* (L.) Osbeck] cv. Valencia late were harvested at veraison from a local orchard in Castellaneta (Italy), selected for uniformity of size and absence of symptoms of any disorders, and immediately processed. Fruit were surface-sterilized with a 2% commercial bleach solution for 2 min, washed with tap water and air-dried at room temperature.

2.2. Testing of salts as resistance inducers

Oranges were wounded once (5 mm depth × 3 mm wide) with a sterile nail-head along the equatorial axis. For each treatment, 30 μL of 3% (w/v) SB or SC solutions were introduced into each wound. Fruit treated with sterile distilled water were used as a control. Treated fruit were placed in a tray, which was then wrapped into a plastic bag. After 48 h of incubation at 20 °C and high relative humidity (RH, 90–95%), another wound was made approximately 5 mm away from the previous one. This wound was air-dried and inoculated with 10 μL of a 10^4 conidia mL^{-1} suspension of *P. digitatum*. Each treatment had 3 replicates made up of 4 oranges each. Replicates were again wrapped in a plastic bag (90–95% RH) and maintained at 20 °C for two weeks. The incidence of decay (percentage of infected wounds, %) and disease severity (lesion diameter, mm) were recorded. The whole experiment was performed twice.

2.3. Tissue sampling for extractions

Fruit were individually wounded with a sterile nail (3 mm wide × 5 mm deep) at eight points on the equatorial surface. Samples were designated as follows: (i) unwounded fruit; (ii) fruit wounded and treated with water; (iii) fruit wounded and treated with SB; (iv) fruit wounded and treated with SC. In each wound, 30 μL of 3% salt solutions or sterile distilled water were applied. For each treatment fruit were randomized and arranged into 5 lots for tissue excision at different time intervals (0, 12, 24, 48, 72 h). Each lot was made up of 3 replicates and each replicate consisted of 4 fruit. The whole experiment was repeated twice. Fruit were arranged in plastic boxes, which were then individually wrapped into plastic bags (90–95% RH) and 20 °C for 72 h. At the established time intervals, tissue cylinders (5 mm) from each lot were excised from the inoculation site. The excised tissues were rapidly frozen in liquid nitrogen, mixed and ground to a fine powder using a commercial blender, lyophilized using a freeze-dryer and pump (VaCo 10-D-N2, Zirbus Dry Technology GmbH, Germany) and stored at −80 °C until use for enzyme, gene expression and metabolic assays.

2.4. Enzyme assays

From each sample and sampling time, 10 g of fine tissue powder were homogenized with 50 mmol sodium acetate buffer pH 5.6 (1:1, w/v), centrifuged (15 min at 10,000 × *g* and 4 °C) and the supernatant filtered through filter paper by a Buchner funnel. Proteins were precipitated in 60% acetone ($v\,v^{-1}$) at −20 °C and the resulting pellet, following centrifugation (30 min at 10,000 × *g* and 4 °C), was washed 3 times with 60% cold acetone. The pellets were dried, resuspended in 2 mL of 50 mmol sodium acetate buffer (pH 5.6) and kept at −20 °C until use. The protein concentration was determined according to Bradford (1976) with the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd., USA).

Chitinase activity was assayed using dye-labeled carboxymethylchitin-RBV (Loewe Biochemica GmbH, Germany) following the method of Wirth and Wolf (1990). The assay was carried out by mixing 100 μL of protein extracts, 100 μL of CM-chitin-RBV and 200 μL of 50 mmol phosphate buffer (pH 6.4). After 2 h of incubation at 37 °C, the reaction was stopped with 100 μL of 2 N HCl, cooled, centrifuged (10,000 × *g*) and the absorbance of the supernatant measured at 550 nm (Multiskan EX, Labsystem). Chitinase activity was calculated according to Wirth and Wolf (1990) and expressed in international units (U) μg^{-1} of total protein. One U is defined as the amount of enzyme required to catalyze the formation of 1 nmol min^{-1} of product.

β-1,3-Glucanase activity was determined following the method of Abeles and Forrence (1979), by incubating 62.5 μL of protein extracts for 2 h at 37 °C in 62.5 μL of 4% (w/v) laminarin. The reaction was stopped by adding of 375 μL of 3,5-dinitrosalicylic acid (DNS) and by heating the sample in boiling water for 10 min and then rapidly cooling it in ice. The absorbance of each sample was measured at 492 nm (Multiskan EX, Labsystem) and activity values reported as μmol glucose equivalents μg^{-1} of total protein min^{-1}.

Peroxidase activity was determined using guaiacol as substrate (Hammerschmidt et al., 1982). The reaction mixture, consisting of 100 μL of crude extract and 100 μL of 50 mmol sodium acetate

buffer pH 5.6 (10 mmol of guaiacol and 10 mmol H_2O_2), was incubated for 60 s at room temperature. The increase in absorbance at 470 nm was spectrophotometrically assayed (Beckman DU 640 Spectrophotometer, Corona, CA, USA) and the enzyme activity was expressed as U μg^{-1} protein s^{-1}.

PAL activity was determined according to Beaudoin-Eagan and Thorpe (1985) with some modifications. The reaction mixture consisted of 100 μL of crude extract and 100 μL of l-phenylalanine 0.1 mol in 0.1 mol borate buffer pH 8.8. After 3 h of incubation at 30 °C, the reaction was stopped by adding 100 μL of 6 N HCl. The reaction mixture was cooled in ice for 5 min and centrifuged 10,000 × *g* for 5 min. The amount of cinnamic acid produced was measured spectrophotometrically at 290 nm and PAL activity was expressed as ng of cinnamic acid μg^{-1} of total protein h^{-1}.

2.5. Gene expression analyses

Total RNA was extracted from orange peel tissues using RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). RNA yield and purity was determined spectrophotometrically and on an agarose gel (1.5%, w/v). Samples were treated with DNAse-Rnase free RQ1 (1 U μL^{-1}, Promega, Milan, Italy), and stored at −80 °C until needed. Total RNA (100 ng) was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad). Several specific primer pairs targeting different genes involved in the host resistance (Table 1) were designed using the Primer3 Software (http://primer3.sourceforge.net/) on the bases of sequences published in GenBank, and synthesized by Sigma–Aldrich (St. Louis, MO, USA). Primers specific to the constitutively expressed housekeeping gene β-tubulin were used as a control. The cDNA was amplified in Real-time PCR reactions using SYBR Green as fluorescent dye. The amplification mixtures (20 μL) contained: 10 μL 2× iQ SYBR Green Supermix (Bio-Rad), 0.5 μL of reverse and forward primers (10 pmol μL^{-1}), 8 μL of nuclease-free water and a 1 μL of cDNA. In negative-control samples, cDNA was replaced by sterile water or total RNA to detect possible cross-contaminations and prove the complete removal of genomic DNA. PCR amplification conditions were: 95 °C for 5 min and then 40 cycles of 94 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. Fluorescence was monitored at each PCR cycle during the extension phase at 72 °C. Amplifications were performed in 96-wells reaction plates using a iCycler iQ thermal cycler (Bio-Rad). Relative normalized fluorescence (DRn) and quantification cycles (Cq) (Bustin et al., 2009) were automatically generated by the iCycler iQTM associate software (Real-Time Detection System Software, version 3.0). Melting curves of real-time PCR products were evaluated from 55 °C to 95 °C to confirm the amplification of single PCR bands. The following cycling conditions were utilized: initial denaturation for 5 min at 95 °C, cooling to 55 °C and melting from 55 °C to 95 °C with a 0.5 °C transition rate every 10 s.

To evaluate, for each gene, the range of concentrations in which target RNA and Cq values were linearly correlated and to determine the reaction efficiency, specific reactions were conducted using cDNA synthesized from serially diluted (1000–0.1 ng) RNA samples. Relative expression of the 4 genes was evaluated by using the ΔΔCq method (Livak and Schmittgen, 2001). Relative expression was calculated according to the following formula: $2^{(-\Delta\Delta Cq)}$, where ΔCq = (average Cq of target gene − average Cq of housekeeping gene) and ΔΔCq = (average ΔCq of untreated sample − average ΔCq treated sample). Data were reported as fold relative expression transformed to $\log_2$. In particular, level of change (*i.e.*, either increase or decrease) in gene expression was categorized based on the following range in $\log_2$ transformed ratios: "low" ≥−1.0 to ≤1.0; "medium" ≥−2.0 to <−1.0, or >1.0 to ≤2.0; "high" <−2.0, or >2.0. The relative expression values were automatically generated by entering Cq values from housekeeping and target genes into special Microsoft® Excel spreadsheets provided by Bio-Rad.

2.6. Metabolic analyses: phytoalexins and sugars

Phytoalexins were extracted according to Ballester et al. (2010) with some modifications. In particular, citrus powdered tissues (150 mg) and 1.5 mL of methanol (80%) were mixed well and vortexed in 7 mL Bijou tubes. Samples were immersed in ice for 60 min and vortexed once again to prevent layering. The samples were then passed through a 0.22 μm syringe filter. The extracts were immediately submitted to HPLC analysis. Citrus peel extracts (5 μL) were injected in an Agilent 1200 series HPLC instrument (Agilent, Berks, UK) equipped with a Waters Spherisorb ODS2 Column (5 μm, 4.6 mm × 250 mm diameter). The mobile phase A was made of double distilled water containing 3% glacial acetic acid; the mobile phase B of acetonitrile with glacial acetic acid (3%). The elution gradient to obtain correct separation of the 3 different phytoalexins at a solvent flow of 1 mL min^{-1} was: 0–10 min, 94% (A) and 6% (B); 10–30 min, 94% (A) and 6% (B); 30–35 min, 82% (A) and 18% (B); 35–50 min, 67% (A) and 33% (B); 50–55 min, 42% (A) and 58% (B); 55–60 min, 94% (A) and 6% (B). The phytoalexins were detected using a fluorescence light detector (FLD, PMTGain 10, Agilent) at excitation and emission wavelengths of 340 and 425 nm, respectively. The column was set at room temperature and the temperature of the autosampler held at 4 °C. The phytoalexins were identified by comparison with the retention time of authentic standards of scoparone, scopoletin, and umbelliferone (Sigma–Aldrich) and quantified by peak area comparison using standard curves built up with authentic calibration standards ranging from 0.01 to 1 mg mL^{-1}.

Fructose, sucrose, and glucose were extracted and measured as described by Chope et al. (2007) with slight modifications. The sugars were extracted from 150 mg of freeze-dried citrus powder using 3 mL of 62.5:37.5 HPLC grade methanol:water (v v^{-1}) and vortexed to mix thoroughly. The samples were incubated at 55 °C in a shaking water bath for 15 min and agitated for 20 s every 5 min to prevent layering and then left to cool. The cooled samples were then passed through a 0.2 μm syringe filter. The extract was then stored at −40 °C until use. Extracts were diluted 1:10 (v v^{-1}) with HPLC grade water immediately before analysis and measured using an HPLC system (Dionex, CA, USA) comprising a P580 pump and GINA 50 autosampler. The diluted extracts (20 μL) were injected into a Rezex RCM monosaccharide Ca^+ column of 300 mm × 7.8 mm diameter, 8 μm particle size (Phenomenex, CA, USA; Part No. 00H-0130-K0) with a Carbo-Ca^{2+} security guard cartridge of 4 mm × 3 mm diameter (Phenomenex; Part No. AJ0-4493). The mobile phase was HPLC grade water at a flow rate of 0.6 mL min^{-1}. Column temperature was set at 75 °C using a Dionex STH column thermostat. An evaporative light scattering detector (ELSD 2420, Waters, MA, USA) connected to the Dionex system using a UCI-50 universal chromatography interface detected the eluted carbohydrates. ELS was chosen as the preferred method of detection due to greater baseline stability and sensitivity as compared to conventional detection by refractive index (Terry et al., 2005). Sugar concentrations were calculated against authentic calibration standards of fructose, glucose, and sucrose ranging from 0.2 to 1 mg mL^{-1} (Sigma, Dorset, UK). Chromatograms were analyzed using Chromeleon Version 4.6 software (Dionex). Data were reported as mean of five time points.

2.7. Statistical analysis

Data were subjected to ANOVA (one-way analysis of variance) using the statistical software package Statistics for Windows (StatSoft, Tulsa, OK, USA). Percentage data were arcsine-square root transformed to normalize variance before ANOVA analysis, but in the graphs untransformed percentages were reported. Significant differences ($P \leq 0.05$) were identified by the General Linear Model

Table 1
Selected primers used in the study. Primers targeted constitutively gene (β-tubulin) or genes involved in defense mechanisms (chitinase, β-1,3-glucanase, PAL, peroxidase).

Primers	Sequences (5′–3′)	Target genes	Accession no.
Tubulin F	GGTGCAAATCCCACCATGAA	β-Tubulin	AF052608
Tubulin R	TGGTGTCACTTGCTGCTGCCTGA		
Chit 2R	CATAACTGGGTGCACATTGG	Chitinase	AF090336
Chit 2F	GAATGCTGCCAAGGCTTATC		
Gluc 2R	ATGGCGTCAAAAAGACTTCG	β-1,3-Glucanase	AJ000081
Gluc 2F	ATTCGCTTCCTCAACGAAAA		
PAL 1F	GCTCATGTTTGCCCAATTTT	PAL	DQ088064.1
PAL 2R	AGAAATTGGAGCTCGGAACA		
POX 1R	TCTGCAAGGGGGTAACAAAC	Peroxidase	AJ582678.1
POX 2F	GCAAAGGTGGACTTTTTGGA		

(GLM) procedure using the Duncan's Multiple Range Test (DMRT). In linear graph data ± standard error of mean were reported (SEM).

3. Results

3.1. Testing of salts as resistance inducers

Green mould incidence and severity on oranges treated or untreated with salts are reported in Fig. 1. Both treatments were able to significantly control the disease incidence (Fig. 1A). In particular, SC completely inhibited the disease until 5 d post-inoculation (DPI) and maintained a significant reduction up to 7 DPI, whereas SB significantly controlled disease incidence all through the incubation period. Similarly, with regard to disease severity (Fig. 1B), a significant reduction of lesion diameters, as compared to the control, was recorded for all treatments, with no significant differences between treatments.

3.2. Enzyme activities

Wounding itself caused an increase in the chitinase enzyme family activity, as compared with the unwounded control, particularly at 12 h of incubation (Fig. 2A).

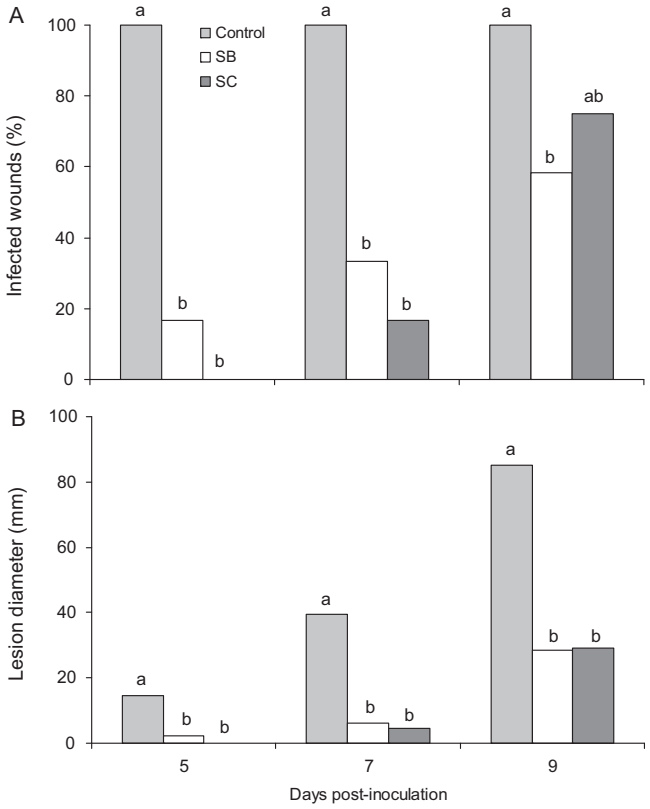

Fig. 1. Disease incidence (% infected wounds) and severity (lesion diameter, mm) caused by *Penicillium digitatum* on "Valencia late" oranges treated or untreated with sodium carbonate (SC) and sodium bicarbonate (SB). For each time assessment, columns marked with the same letters are statistically not different according to DMRT ($P \leq 0.05$).

However, in the presence of SC and SB, this enzyme activity was lower than the one recorded in unwounded and wounded controls.

β-1,3-Glucanase activity in tissues treated with SC and SB and sampled at 12 h was 2.2 and 1.6 fold higher compared to unwounded control, respectively. In addition, in tissues treated with SB and SC, β-1,3-glucanase activity increased with time, reaching a maximum at 24 and 48 h, respectively. In particular, at 48 h the activity of β-1,3-glucanase in tissues treated with SC increased 1.7-fold as compared to all other treatments (Fig. 2B).

The peroxidase activity was higher in wounded tissue treated with salts or water as compared to the unwounded control, and showed an increase with time, reaching its maximum at 12–24 h (Fig. 2C). The highest activity of peroxidase was detected in SB-treated tissue, where it was 1.7-fold higher compared to unwounded controls. However, in tissues treated with SC, the peak of activity (1.6-fold) was reached 12 h earlier than in SB-treated tissues.

Changes in PAL activity are shown in Fig. 2D. SB treatment induced an increase in PAL activity reaching the maximum level at 12 h, as compared to the controls. In particular, the activity was increased by 1.4- and 2.3-fold as compared to wounded and unwounded controls, respectively. An increase in PAL activity was also observed with time in tissues treated with SC, with the peak at 48 h corresponding to a 1.4- and 1.9-fold increase, compared to wounded and unwounded controls, respectively.

3.3. Gene expression analyses

Linear equations, determination coefficients (R^2) and reaction efficiencies for all primer pairs tested are reported in Table 2. Cq values and the logarithm of RNA concentrations (ng) were linearly correlated for each of the examined genes in the range 1000–0.1 ng. A similar reaction efficiency, included in the optimal range 90–110%, was assessed for the housekeeping gene (β-tubulin) and the four target genes. As cDNA synthesized from 100 ng of total RNA was efficiently amplified, this concentration was utilized in the subsequent real-time PCR reactions. The melting curve analysis showed the presence of a single melting peak for each of the tested genes, indicating that all primer pairs reported in Table 1 amplified a single product with a distinct melting temperature (data not shown). In all negative-control samples, no fluorescent signal was detected, proving that there were no contaminations in the reaction mixtures and that the DNA traces were effectively removed.

Expression patterns of chitinase, β-1,3-glucanase, peroxidase and PAL genes in orange tissues, treated with SC and SB were determined using the $2^{-\Delta\Delta Cq}$ method and normalized by the β-tubulin gene. The fold changes in gene expression, compared to wounded and unwounded control, were $\log_2$ transformed. The quantification was performed at two different time points (12 and 24 h) (Fig. 3).

At 12 h post-salt application, data showed a general increase in the transcript level of the PAL gene, this being significantly up-regulated to a high level when the fruit were wounded and treated by SC and SB, as compared to wounded (14.4- and 5.7-fold, respectively) and unwounded (51.2- and 10-fold, respectively) controls. However, even in wounded tissues treated with water, significant PAL up-regulation was observed as compared to unwounded tissues. In contrast, the other three genes were down-regulated in both treated and untreated tissues, with the exception of the peroxidase gene which was up-regulated in wounded fruit as compared to the unwounded control.

Table 2
Linear equations, determination coefficients (R^2) and reaction efficiencies obtained by plotting cDNA concentrations (log ng) and Cq values experimentally achieved by real-time PCR for genes chitinase, β-1,3-glucanase, peroxidase, PAL, and β-tubulin.

Gene	Linear equation	R^2	Reaction efficiency (%)
β-Tubulin	$Y = -3.291X + 32.352$	0.99	101.3
Chitinase	$Y = -3.177X + 39.138$	0.993	106.4
β-1,3-Glucanase	$Y = -3.374X + 38.725$	0.997	97.9
PAL	$Y = -3.145X + 32.867$	0.995	107.9
Peroxidase	$Y = -3.375X + 40.067$	0.999	97.8

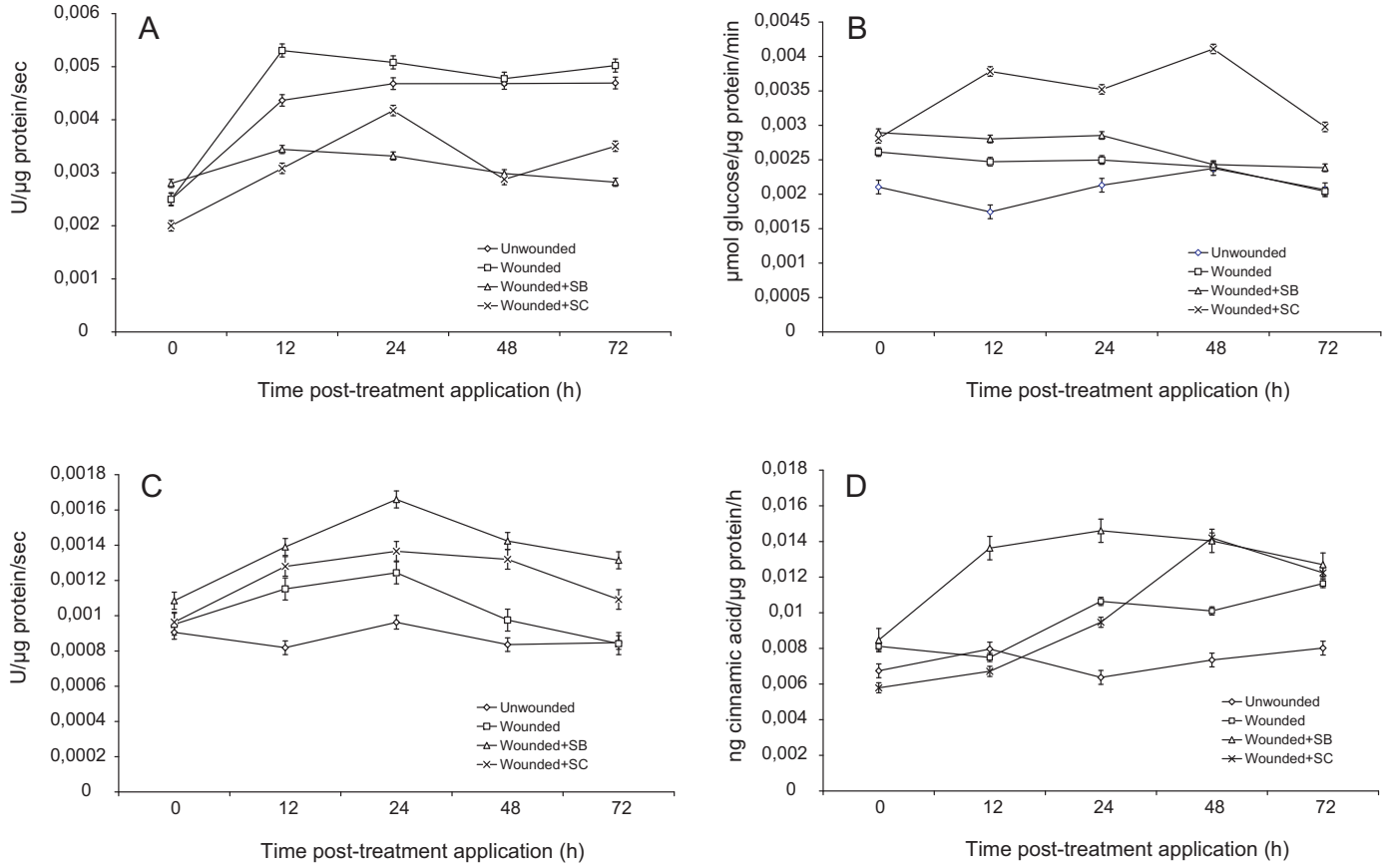

Fig. 2. Time course of chitinase (A), β-1,3-glucanase (B), peroxidase (C), and PAL (D) activity in extracts from "Valencia late" orange peel unwounded, wounded, wounded + sodium carbonate (SC) and wounded + sodium bicarbonate (SB). Data are the mean of two experiments. Bars represent standard error of mean (SEM).

At 24 h post-salt application, PAL encoding gene continued to be significantly up-regulated at a high (18.9-fold) and medium (3.2-fold) level when fruit were treated with SB and SC, respectively, as compared to the unwounded control. However, when the salt effect was considered alone, only SB seemed to maintain its inductive effect on PAL expression, although at a low level (1.5-fold), as compared to wounded controls.

3.4. Metabolic analyses: phytoalexins and sugars

The three main phytoalexins (scoparone, scopoletin, and umbelliferone) of citrus peel tissues were detected in extracts using a fluorescent light detector and the above mentioned HPLC conditions. The retention times for the standards corresponding to umbelliferone, scopoletin, and scoparone were 35.8, 37.4, and 42.4 min, respectively. The concentration of those phytoalexins in the samples was calculated by interpolation from a compound-specific standard curve. The accumulated levels of the phytoalexins were determined at various time intervals (Fig. 4). HPLC analysis showed increased amounts of the compounds in salt-treated tissues, as compared to those recorded in the two controls, with the accumulation of scoparone being the most pronounced. The amount of scoparone increased with time: after 72 h of treatment, SB- and SC-treated tissue showed a 4.4- and 4.5-fold increase as compared to wounded controls, respectively. An increase of scopoletin accumulation was observed after 24 h in tissues wounded and treated with SB and SC, by 1.74- and 1.9-fold respectively, as compared to wounded controls. The highest induction of umbelliferone was detected after 24 h in SB and SC-treated tissue by 2.5- and 3.9-fold, respectively, as compared to the wounded control.

Since, for each treatment, no significant differences among sampling times were observed, mean fructose, glucose, and sucrose concentrations in citrus peel tissues were calculated and reported in Fig. 5. However, slight differences were detected among the different treatments. In fruit wounded and treated with SB, fructose concentration was not significantly different as compared to wounded control, whereas a significant reduction (9.8%) was detected as compared to the unwounded control. In particular, the mean concentrations of fructose were 82.8, 77.1, 72.9, and 77.4 mg g^{-1} DW in unwounded, wounded, wounded + SB, and wounded + SC fruit, respectively.

A significant difference in glucose concentrations was found in fruit wounded and treated with SB, showing 23.6% and 13.4% reduction as compared to unwounded and wounded controls, respectively. In the case of SC, the glucose concentration was significantly reduced by 14%, as compared to unwounded controls, whereas no significant reduction was detected compared to the wounded control. In particular, glucose concentrations were 110.9, 100.6, 87.3 and 96.8 mg g^{-1} DW in unwounded, wounded, wounded + SB, and wounded + SC fruit, respectively.

In contrast, sucrose concentrations were significantly higher in the presence of SB with a 24.6% increase as compared to unwounded controls. In particular, sucrose concentrations were 59.4, 65.5, 83.9, and 68.8 mg g^{-1} DW in unwounded, wounded, wounded + SB and wounded + SC fruit, respectively.

4. Discussion

Carbonate and bicarbonate salts, alone or in combination, offered some degree of protection against postharvest pathogens of citrus fruit (Palou et al., 2008; Youssef et al., 2012a,b). As reported in the literature, the growth inhibition is probably due to a direct effect on the pathogen, consisting in a reduction of fungal cell turgor pressure with a consequent collapse and shrinkage of hyphae and spores and an inability to sporulate and produce extracellular degrading enzymes (Fallik et al., 1996). In addition, although much of the previous research indicated that pH plays an important role in the antifungal activity of salts, Smilanick et al. (1999) reported great differences in *P. digitatum* control efficacy by salts having the same pH, suggesting the existence of an indirect effect on the host. Moreover, Venditti et al. (2005) found alkalization and structural changes of the albedo and increased levels of scoparone in wounded tissue treated by SC. In a recent study, D'Aquino et al. (2013) showed that SB was able to induce changes in the epicuticular wax morphology and distribution on the fruit surface. In the present investigation, we found that when applied in a wound different from but close to the one inoculated with the pathogen, SC and SB were effective in controlling disease development, further supporting the putative role of salts as resistance inducers. In order

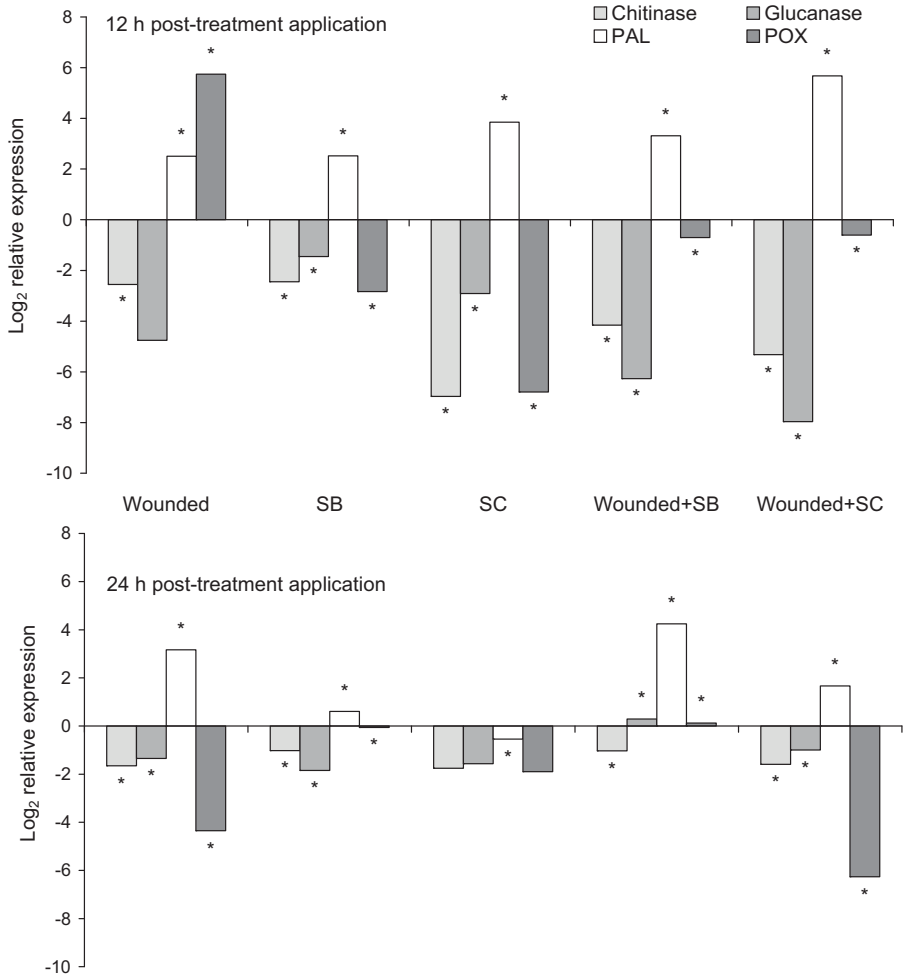

Fig. 3. Relative expression (log_2 transformed) of chitinase, β-1,3-glucanase, peroxidase, and PAL in "Valencia late" orange peel unwounded, wounded, wounded + sodium carbonate (SC) and wounded + sodium bicarbonate (SB) at 12 and 24 h after treatment application. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized using the β-tubulin gene. Values are the mean of two experiments. Columns with an asterisk are statistically different according to DMRT ($P \leq 0.05$) as compared to their control.

to confirm this hypothesis at a chemical, biochemical, and molecular level, changes in chitinase, β-1,3-glucanase, peroxidase, and PAL activity and expression, as well as in phytoalexin (scoparone, scopoletin, umbelliferone) and sugar (glucose, fructose, sucrose) contents of "Valencia late" oranges were studied. The analysis of the accumulation kinetics of the selected enzymes in treated tissues showed that SC and SB induced an increase in the activity of almost all tested enzymes as compared to the controls. The activity of chitinase was the only one reduced, probably in relation to the high pH (11.4 and 8.6, for SC and SB, respectively) of the two salt solutions. Indeed, it is known that the optimal pH for chitinase activity is usually acidic (Koga et al., 1999). Moreover, the low chitinase activity reported here is in agreement with the results obtained by Ballester et al. (2010) in the flavedo of cured orange fruit. Concerning the other enzymes, the increase in β-1,3-glucanase activity is particularly interesting. Glucanases can degrade the fungal cell wall mainly composed of β-1,3-glucan, playing a role in delaying growth, spore germination and germ tube elongation, and, therefore, decreasing the incidence of *P. digitatum* infection (Ballester et al., 2010). Similarly, Droby et al. (2002) highlighted β-1,3-glucanase induction in the flavedo of citrus fruit treated with the yeast *Candida oleophila*. Finally, *Aurebasidium pullulans* has been shown to be able to induce a significant increase of this enzyme activity in wounded apples (Ippolito et al., 2000; Castoria et al., 2001). Concerning peroxidase, a peak of activity was detected in SB-treated tissue at 24 h and in SC-treated tissues starting from 12 h post-treatment, thus suggesting a role of peroxidase in the beneficial effect of the postharvest salt treatment. The results obtained herein are in agreement with other findings, which demonstrate an increased peroxidase activity in citrus fruit elicited by UV irradiation (Droby et al., 1993) and in response to *P. digitatum* infection (Ballester et al., 2006). As a consequence, fruit might be less susceptible to fungal invasion because of cell wall reinforcement (lignin formation) and increases in tissue antioxidant ability (Hammerschmidt et al., 1982). Among all the analyzed enzymes, PAL is the one that showed the largest induction in response to salt treatment. In particular, at 12 h from its application, SB caused the strongest induction of PAL activity (2.3-fold) as compared to all other treatments, while in SC-treated tissues, PAL activity reached its maximum at 48 h. Thus, in the presence of SB, treated tissues might react more quickly than in the presence of SC, and as a result, they might be more resistant to pathogen infection since an earlier response is more effective in limiting tissue colonization. PAL is considered important in host resistance mechanisms, since it is involved in several metabolic pathways including the phenylpropaniod one, from which scoparone and scopoletin are synthesized.

As confirmation, the expression levels of chitinase, β-1,3-glucanase, peroxidase, and PAL genes were analyzed in the presence/absence of salt solutions. When relative expression was determined at 12 h post-treatment, wounding, SC, and SB as a stand alone treatment were able to significantly increase mRNA accumulation for the PAL gene as compared to the unwounded

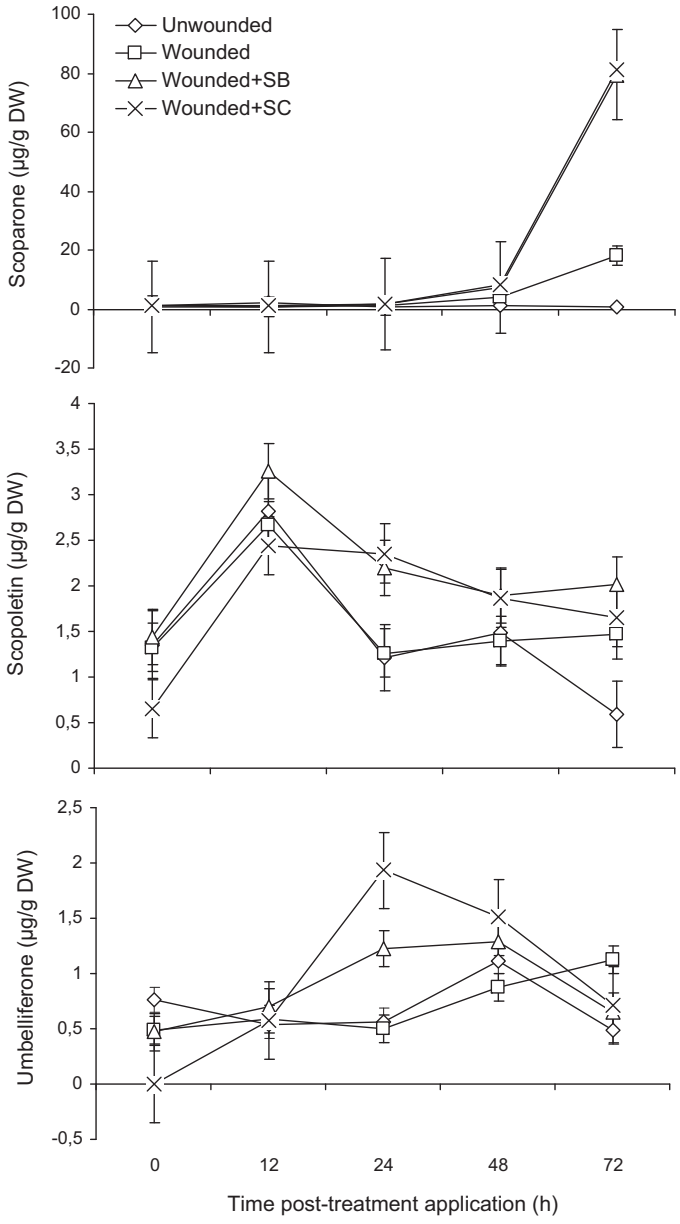

Fig. 4. Scoparone, scopoletin, and umbelliferone concentration ($\mu g\,g^{-1}$ dry weight) in "Valencia late" orange peel unwounded, wounded, wounded + sodium carbonate (SC) and wounded + sodium bicarbonate (SB). Data represent the mean of two experiments. Bars represent standard error of mean (SEM).

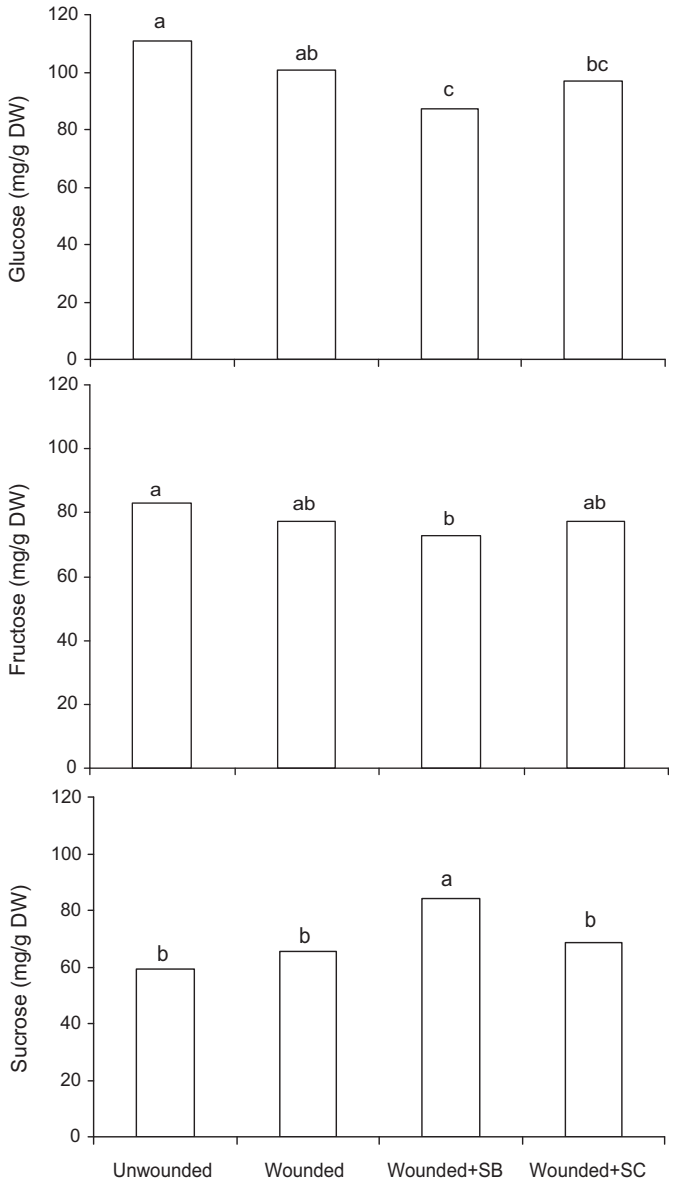

Fig. 5. Fructose, glucose, and sucrose concentration ($mg\,g^{-1}$ dry weight) in "Valencia late" orange unwounded, wounded, wounded + sodium carbonate (SC) and wounded + sodium bicarbonate (SB). Data represent the mean of two experiments of 5 time points each. Columns marked with the same letters are not statistically different according to DMRT ($P \leq 0.05$).

control. This finding seems to suggest that salt application up-regulates a metabolic pattern similar to the one involved in the response to general stresses such as wounding (Vilanova et al., 2013). This induction might result in natural host defence mechanisms, although only the combination of wounding + SC had a synergic effect. Similarly, Sanzani et al. (2010) reported that the expression of defence genes after wounding was enhanced by treating apple fruit with exogenous quercetin. Nevertheless, wounding alone or in the presence of SB, were the only treatments that further increased the inductive effect 24 h from application. These results suggest that SC induces an earlier PAL up-regulation as compared to SB, since it reached its maximum induction level at 12 h after salt application. A similar behaviour has been described for stress-related genes in salt-treated *Arabidopsis* seedlings (Strizhov et al., 1997), salt-stressed rice (Kawasaki et al., 2001) and *Erwinia amylovora* challenged apples (Norelli et al., 2009). Furthermore, a correspondence with enzyme activity was found. The combination wounding + SB seems to postpone PAL maximum up-regulation at 24 h, thus prolonging the salt protective effect. It has also been reported that stress-inducible genes reach their maximum expression some time after treatment, depending on the gene itself and the stress experienced (Strizhov et al., 1997). Finally, the expression of chitinase, β-1,3-glucanases, and peroxidase genes proved to be generally down-regulated by all treatments. These results, in contrast with the observed enzyme activities, may be ascribed to a possible earlier induction (before 12 h post-treatment) that would have required an earlier tissue sampling. Moreover, it has to be considered that, while enzyme assays included all the possible isoforms of an enzyme, molecular assays focus on a particular form, whose nucleotide sequence is available in public databases.

In citrus, PAL genes are involved in the synthesis of antifungal compounds such as the phytoalexins scoparone and scopoletin, whose concentration increases in the peel in response to biotic

and abiotic stresses (Kim et al., 1991; Droby et al., 2002; Ballester et al., 2010). The HPLC analysis of the peel extracts confirmed increased amounts of scoparone, scopoletin, and umbelliferone, as compared to the levels found in the control tissues, with the accumulation of scoparone being the most pronounced. Scoparone accumulated with time, with a maximum at 72 h from treatment with both SB and SC, confirming previous findings (Venditti et al., 2005; Dore et al., 2010). Whereas, a significant increase in scopoletin and umbelliferone content, as compared to the controls, was observed after 24 h in salt-treated tissues. A similar accumulation pattern of the three phytoalexins in *C. oleophila*-challenged grapefruit peel tissue has also been reported (Droby et al., 2002). Brown (1985) suggested that umbelliferone might be a precursor of scopoletin, which, in turn, is thought to be a precursor of scoparone (Rodov et al., 1992). This might explain the lowest level of umbelliferone and scopoletin detected in our salt-treated tissues as compared to scoparone content. Furthermore, we were able to detect the induction of scopoletin and umbelliferone by SC and SB, even when present at low amounts, thanks to an HPLC protocol set up to chromatographically separate and thus accurately quantify the three phytoalexins using fluorescence light. Indeed, unlike scoparone, little attention has been paid to scopoletin and umbelliferone detection and quantification in previous studies, possibly because of their inefficient extraction from the peel tissues, usually conducted using less polar solvents such as dichloromethane.

HPLC results also demonstrated a change in sugar content of salt-treated citrus tissues. Similarly, it has been reported that sodium benzoate significantly affected the soluble sugars contents of potato tubers (Yaganza et al., 2003). In particular, in our samples the content of sucrose significantly increased, due to SB application, as compared to unwounded and wounded control. This increase may determine a reduction in water activity, causing unfavourable conditions for pathogens to establish infection on citrus peel. Moreover, it has been reported that phenolics and anthocyanins increase rapidly in the skin of grape berries one week after the start of soluble sugar accumulation (Pirie and Mullins, 1980) depending on the availability of phenylalanine, which is synthesized from sugars through the shikimate pathway (Hradzina et al., 1984). Thus, sugar accumulation might provide the substrate needed for the synthesis of various secondary metabolites including phytoalexins (Jackson, 2008). In our samples, salts could have contributed to trigger PAL and consequently the shikimate pathway, as suggested by biochemical and gene expression results and by metabolic analyses.

In conclusion, a detailed study of the ability of SC and SB to induce resistance in oranges has been carried out. The accumulation of phytoalexins and carbohydrates which are metabolites able to control, directly or indirectly, pathogen infection, reinforces the idea that PAL is involved in the mode of action of tested salts. This response seems to sum up to citrus natural reaction to wounding, enhancing its protective effect and contributing to a successful defence against pathogens. This study suggests that induced resistance should be considered as an important aspect of the multiple mechanisms of sodium salts for controlling postharvest decay of citrus fruit.

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